AD ______________

GRANT NUMBER DAMD17-97-1-7331

TITLE: Elucidating the Role of CaMKK in Cell Cycle and Cell Fate Using a C. elegans Model

PRINCIPAL INVESTIGATOR: Ethan E. Corcoran

CONTRACTING ORGANIZATION: Duke University
Durham, North Carolina 27710

REPORT DATE: July 1999

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
distribution unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE			Form Approved OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 1999	Annual (1 Jul 98 - 30 Jun 99)

4. TITLE AND SUBTITLE	5. FUNDING NUMBERS
Elucidating the Role of CaMKK in Cell Cycle and Cell Fate Using a C. elegans Model	DAMD17-97-1-7331
6. AUTHOR(S) Corcoran, Ethan E.	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Duke University Durham, North Carolina 27710	8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012	10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT	12b. DISTRIBUTION CODE
Approved for public release; distribution unlimited	

13. ABSTRACT *(Maximum 200 words)*

The mammalian calmodulin-dependent protein kinase kinases (CaMKKs) have been shown to phosphorylate and activate the calmodulin-dependent protein kinases I and IV (CaMKI, CaMKIV), leading to transcription of a variety of genes important in cell cycle regulation. Using a *C. elegans* homologue (ceCaMKK), we are investigating the importance of this proposed cascade to cell cycle, cell fate and development in the context of a well defined multicellular organism. By generating transgenic worms with reporter proteins controlled by the ceCaMKK promoter, gene expression has been demonstrated in the excretory cell, vulval muscle cells and several neurons of adult hermaphrodites, in the hypodermal cells of L1/L2 larvae, and in several male-specific tail cells. To examine this pathway biochemically, we have cloned the ceCaMKK and ceCaMKI cDNAs, and produced recombinant proteins by prokaryotic expression methods. Both mammalian and *C. elegans* CaMKKs can phosphorylate either species' CaMKI homolog specifically on the acitvation loop (T177 in human, T179 in *C. elegans*) *in vitro*. These results indicate a functional homology between the mammalian and *C. elegans* calmodulin dependent kinases, and demonstrate the existance of a calmodulin dependent kinase cascade in the worm. Further work remains to reveal the biological functions of this signaling.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer	11
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

____ Where copyrighted material is quoted, permission has been obtained to use such material.

____ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

EC Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

____ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

✓ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

EC In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

EC In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

____ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

PI - Signature 30 July 99
Date

Elucidating the Role of CaMKK in Cell Cycle and Cell Fate Using a *C. elegans* Model

Annual Report for Grant DAMD17-97-1-7331
July 1, 1998 to June 30, 1999

Ethan E. Corcoran

Table of Contents

Introduction

Calcium and calmodulin (CaM) are ubiquitous signaling molecules, implicated a wide variety of cellular functions including cell cycle control. Ca^{2+}/CaM signals are critical in G_0 reentry, G_1/S, G_2/M, and metaphase transitions in a variety of organisms (1). Recently, a novel kinase cascade has been proposed connecting the action of CaM dependent kinase kinases (CaMKK) to transcription of key factors in cell cycle control, through the phosphorylation and activation of calmodulin dependent kinases I and IV (CaMKI and CaMKIV) (2-7). In addition, mammalian CaMKK has now been implicated in the regulation of protein kinase B and of the mitogen-activated protein kinase (MAPK) cascade, both of which have significant established roles in cell cycle control and apoptosis (8-9). This project was designed to assess the biological relevance of these relationships and of the CaMKK itself. Using a *Caenorhabditis elegans* model, any perturbation of normal cell division or cell fates can be identified (10-12). We have now cloned cDNAs for *C. elegans* isoforms of both CaMKK and CaMKI (ceCaMKK and ceCaMKI), examined their biochemical homology using recombinantly expressed proteins, and used transgenic techniques to determine the developmental expression pattern of ceCaMKK. We are currently pursuing the downstream effectors of this cascade through an in vitro screen for kinase substrates of either protein, and attempting to isolate null mutants for these genes. Understanding this kinase cascade, from protein interactions to biological consequences, may help unravel the essential functions of calcium signals in the cell cycle.

Results and Discussion

As indicated in last years report, the first project objective of cloning ceCaMKK has been completed. While we have not empirically confirmed the sequence of the last 80 bp of the 5' end, a recent report successfully used rtPCR to clone the predicted C05H8.1 gene (13). Unfortunately, while this study does verify the 5' start sequence, the 3' primers included an assumed TAA stop codon. This oversight led to the conclusion that the transcript is identical to the predicted open reading frame, and missed the final two exons of the gene. Since our cloning strategy involved hybridization screening of a cDNA library, and did not assume the location of the stop codon, we have avoided this pitfall (14). Interestingly, the only biochemical difference demonstrated so far between the full length ceCaMKK and this truncated C05H8.1 is autophosphorylation of the truncated form (13).

The second objective in this project, determining expression patterns and isolating null mutants, has presented the greatest difficulties to date. In last year's report, the developmentally regulated expression pattern for hermaphrodite worms had been examined using transgenic methods (15). Expression is observed in the adult vulval muscle cells, amphid sensory neurons and the excretory cell, as well as in L1 hypodermal cells. Using the same transgenic constructs, the male nematodes' expression pattern has been shown to include the excretory cell, amphid sensory neurons, and several male specific tail cells, believed but not yet confirmed to be neurons. Larval males have not been examined. To extend these results, transgenic constructs that include the entire ceCaMKK gene (promoter and coding regions) in fusion with GFP are being made. These constructs will allow the subcellular localization of the protein to be determined, as a function of development and stimulation of the relevant neurons in adults.

The difficulty with these methods for determining expression has been an inability to confirm the patterns using a second technique. In-situ hybridization has been attempted using standard methods and three different gene specific DNA and RNA probes without success, most likely because the expression levels of the ceCaMKK are so low (16-17). Our labs are able to use these methods for more strongly expressed signals, such as RNAs induced by cadmium exposure (18). No specific antibody is yet available, so immunohistochemical methods have not been tried. The increasingly frequent use and confirmation of transgenic methods for studying expression patterns makes secondary confirmation of this pattern less important than it was when the project was first proposed. However, being unable to confirm the presence or absence of either RNA message or protein in a worm makes it difficult to determine the success of attempts to use RNAi suppression of the gene.

The second focus of objective two, obtaining and characterizing null mutants of ceCaMKK, is still in progress. Two methods are being employed - PCR screening of mutagenized worm libraries and RNAi. PCR screening of mutant libraries requires the construction of a mutagenized deletion worm library, which can be generated using well-established techniques (19). Once the worms have been mutagenized, they are split into small groups, grown for one generation, and divided into a freezer stock and a DNA stock. The DNA stock is used to make genomic templates for PCR reactions, while the freezer stock is saved for the recovery of any relevant worm mutants. A two step nested PCR strategy is used to identify any deletions within the region of interest, which can be followed until a unique strain has been isolated from the mixture. Our lab participated in the construction of such a library in collaboration with four other labs at Duke. PCR screening of this library has not yet yielded any relevant mutants. The same basic strategy has been adopted by the Sanger Center's C. elegans Gene Knockout Program, with the goal of eventually obtaining knockouts of every known worm gene (20). We have requested that they screen their libraries for deletions of the ceCaMKK gene, but, at last report, two libraries had been screened with no positives. The Sanger Center is currently working on methods to improve the library generation and screening procedures, and will continue to search for deletions in the ceCaMKK locus.

The alternate method for obtaining null mutants in a gene involves the poorly understood phenomenon of RNAi. It has been shown for many early larval genes that injection of double stranded RNA effectively abolishes the expression of that gene in the progeny of injected worms(21-23). The effectiveness of the expression block varies with the gene, the dsRNA used, the cells involved and the stage of development. It is most effective during early development, but has been observed to cause adult phenotypes and sometimes persist into another generation (21). We have generated and injected dsRNAs corresponding to the ceCaMKK mRNA, but no phenotype has been observed. This could indicate that there is no developmental consequence of ceCaMKK gene suppression, that the consequence is not obvious without appropriate testing, or that the gene has not been successfully suppressed by the dsRNA. As mentioned previously, we are continuing to work on a method for measuring expression levels *in situ* in order to determine the success or failure of RNAi in this case. The search for null mutant of the ceCaMKK will continue to be a major focus of the project during the next year.

The final objective of the project is to identify other components of the ceCaMKK pathway. As mentioned last year, by BLAST searching the C. elegans genomic sequence, a potential homologue of CaMKI was identified in the predicted gene K07A9.2 (24). The open reading frame appeared to be missing the 5' end, including the ATP binding loop. This may have been due to uncertainty in the genomic sequence 5' to the gene. Initially, the predicted

gene was cloned by rtPCR, and 5' RACE was used to find the remaining 5' region. The 5' RACE products were all short sequences, ending at similar sites only 20 bases upstream of the predicted start. Searching upstream genomic sequences for homology to the 5' end of CaMKI revealed two additional potential exons beginning more than 8 kb 5' of the predicted K07A9.2 start site. By rtPCR, these exons were amplified as part of a complete ceCaMKI mRNA, having 58% amino acid identity to human CaMKI (30% to human CaMKIVa). Nonetheless, the shorter transcript, corresponding to the predicted K07A9.2 gene, includes sequences not found in the longer transcript, has its own start codon, and appears by 5'RACE to have a transcriptional start site. It is possible, therefore, that this gene has two products, one a functional kinase and the other, by truncation of the ATP binding domain, a kinase dead variant. This may be analogous to the mammalian CaMKIV gene, which is known to have multiple products including an active kinase and a truncated form regulated by an internal promoter whose function is unknown (3). We are working on several methods to examine the ceCaMKI gene, including northern blots using probes to the 3' end of the mRNA and affinity purification of a mammalian CaMKI antibody, which by homology has a good chance of recognizing the worm protein but as a polyclonal antiserum reacts with many proteins in worm extracts. In addition, transgenic expression constructs are being generated which will include various regions of the promoter and first introns, to examine the kinase's expression pattern and ascertain if there is an internal promoter which might regulate a second gene product.

The full length ceCaMKI cDNA has been used for recombinant expression to study the protein's biochemistry. It expresses well in bacteria as a GST fusion protein, and can be purified to near homogeneity. When used for kinase assays, it is remarkably similar to human CaMKI. It binds CaM in overlay experiments, is Ca^{2+}/CaM dependent for its kinase activity, and is phosphorylated and activated by either recombinant mammalian CaMKKB or ceCaMKK (25-26). Mutation of threonine 179 to alanine in the activation loop (analogous to T177A in the human form) abolishes phosphorylation by kinase kinases and prevents activation (26). The specific ATP, CaM and peptide substrate binding constants have not yet been determined.

With the identification and expression of the ceCaMKI, the focus for screening the ceCaMKK pathway, objective three, has turned to finding substrates of the kinase cascade. Following the methods successfully applied by Tony Hunter and colleagues in the screen for MAPK substrates (27), we have been developing an *in vitro* screen for protein substrates of this kinase cascade. We have a unidirectional cDNA library based on the lambda-ZAP-XR vector (Stratagene); this type of library has been used for antibody expression screens. The basic strategy involves inducing a growing phage cDNA library with IPTG impregnated nitrocellulose membranes. After 4-6 hours at 30 degrees, these filters are removed and a duplicate filter is placed on each plate for a further 4 hours. The filters bind proteins from the bacterial lysates made by the lytic phage infection, including the expressed cDNA specific to each phage. The filters are blocked, washed and incubated with ATP (to avoid isolating ATP-binding proteins), before they are exposed to activated kinase in the presence of γ-^{32}P-ATP. After further washes to remove unincorporated isotope, the filters are exposed to film, and positives appearing on both duplicate filters can be selected for further screening. The reaction conditions have been adapted to accommodate the calmodulin-dependent protein kinases by including calcium, calmodulin and increased ATP in the reaction buffer and by preincubating the kinase with kinase kinase at high ATP concentrations to activate the kinase. The ceCaMKK has been difficult to purify and is only produced in small quantities, so recombinant mammalian CaMKKB is used to activate

ceCaMKI used for the screen. Samples of the final kinase buffer are very reactive against purified substrates.

To test these methods, a lambda-ZAP (Stratagene) control phage containing a convenient protein substrate sequence as an insert (the 1-117 amino acid fragment of p300, referred to as λ117) (28). Bacteria infected with λ117 have been used in comparison with bacteria infected with wild type phage (λWT) to test conditions for the screen. At this point, λ117 and λWT derived bacterial extracts are easily identified on a gel assay, with a very strong band corresponding to the 1-177 p300 fragment, but when immobilized on filters, the signal from λ117 is difficult to distinguish from λWT. It should be possible to adjust conditions so that the background is sufficiently distinguishable from a positive signal. Using these reagents, screening conditions will be optimized until the signal to background ratio is high enough to permit a full scale screen of the cDNA expression library. Since we are screening a *C. elegans* library, not mammalian, fewer plaques need to be screened for complete coverage of the genome, and any substrate cDNA fragments identified should be more easily cloned using information from the cDNA and genome sequencing projects.

Conclusions

We have identified and cloned a calmodulin dependent protein kinase cascade in *C. elegans*. Using recombinant expression, we have confirmed biochemical homology between the *C. elegans* and mammalian CaMKK and CaMKI, and are continuing to search for targets of the cascade by an in vitro screening method. To define biological functions of this cascade, we are investigating the expression patterns and gene regulation of these kinases and attempting to generate null mutants of these genes using the available reverse genetic techniques. Completion of this project will be a significant advance in our understanding of the biological roles of calcium, calmodulin and calmodulin dependent protein kinases.

Appendices

Key Research Accomplishments

1. Cloning of the *C. elegans* homologue of the calmodulin dependent protein kinase kinase (ceCaMKK)
2. Prokaryotic expression of recombinant ceCaMKK
3. Confirmation of biochemical homology between mammalian CaMKK and ceCaMKK
4. Determination of the developmental and cell specific expression pattern of ceCaMKK in *C. elegans* using transgenic methods
5. Cloning of the *C. elegans* homologue of the calmodulin dependent protein kinase I, ceCaMKI
6. Prokaryotic expression of recombinant ceCaMKI as a GST fusion protein
7. Confirmation of biochemical homology between mammalian CaMKI and ceCaMKI
8. Development of an in vitro screening protocol for finding substrates of the CaM dependent kinases

Reportable Outcomes

None to date.

Acronyms/Abbreviations

ATP	adenosine triphosphate
C. elegans	*Caenorhabditis elegans*
CaM	calmodulin
CaMKI	calmodulin dependent kinase I
CaMKIV	calmodulin dependent kinase IV
CaMKKB	calmodulin dependent kinase kinase B
ceCaMKK	*C. elegans* calmodulin dependent protein kinase kinase
ceCaMKI	*C. elegans* calmodulin dependent protein kinase I
CREB	cyclic adenosine 3',5'-monophosphate response element binding protein
GFP	green fluorescent protein
GST	glutathione-S-transferase
IPTG	isopropyl β-D-thiogalactopyronoside
λ117	lambda-ZAP phage expressing the 1-117 amino acid fragment of p300
λWT	wild type lambda-ZAP phage
MAPK	mitogen-activated protein kinase
MBP	maltose binding protein
PCR	polymerase chain reaction
PKB	protein kinase B
RACE	rapid amplification of cDNA ends
RNAi	double stranded RNA mediated inhibition (see ref 21)

References

1. Means, A.R. (1994). Calcium, calmodulin and cell cycle regulation. FEBS Lett. 347, 1-4.
2. Tokumitsu, H., Enslen, H., Soderling, T.R. (1995). Characterization of a Ca^{2+}/Calmodulin-dependent Protein Kinase Cascade. J. Biol. Chem. 270, 19320-19324.
3. Means, A.R. Ribar, T.J. Kane, C.D., Hook, S.S., Anderson, K.A. (1997). Regulation and properties of the rat Ca^{2+}/calmodulin-dependent protein kinase IV gene and its protein products. Recent Prog Horm Res. 52, 389-406.
4. Sheng, M., Thompson, M.A., Greenberg, M.E. (1991). CREB: A Ca^{2+}- Regulated Transcription Factor Phosphorylated by Calmodulin-Dependent Kinases. Science 252, 1427-1430.
5. Desdouets, C., Matesic, G., Molina, C.A., Foulkes, N.S., Sassone-Corsi, P., Brechot, C., Sobczak-Thepot, J. (1995). Cell Cycle Regulation of Cyclin A Gene Expression by the Cyclic AMP-Responsive Transcription Factors CREB and CREM. Mol. Cell Biol. 15, 3301-3309.
6. Selbert, M.A., Anderson, K.A., Huang, Q., Goldstein, E.G., Means, A.R., Edelman, A.M. (1995). Phosphorylation and Activation of Ca^{2+}-Calmodulin-dependent Protein Kinase IV by Ca^{2+}-Calmodulin-dependent Protein Kinase Ia Kinase. J. Biol. Chem. 270, 17616-17621.
7. Tokumitsu, H., Brickley, D.A., Glod, J., Hidaka, H., Sikela, J., Soderling, T.R. (1994). Activation Mechanisms for Ca^{2+}/Calmodulin-dependent Protein Kinase IV. J. Biol. Chem. 269, 28640-28647.
8. Enslen, H., Tokumitsu, H., Stork, P.J., Davis, R.J, Soderling, T.R. (1996). Regulation of mitogen-activated protein kinases by a calcium/calmodulin-dependent protein kinase cascade. Proc. Nat. Acad. Sci. USA 93, 10803-10808.
9. Yano, S., Tokumitsu, H., Soderling, T.R. (1998). Calcium promotes cell survival through CaM-K kinase activation of the protein-kinase-B pathway. Nature. 396, 584-587.
10. Sulston, J.E., Horvitzm H.R. (1977). Post-embryonic Cell Lineages of the Nematode, *Caenorhabditis elegans*. Dev.l Biol. 56, 110-156.
11. Kimble, J., Hirsh, D. (1979). The postembryonic cell lineages of the hermaphrodite and male gonads in *Caenorhabditis elegans*. Devl. Biol. 56, 32-38.
12. Sulston, J.E., Schierenberg, E., White, J.G., Thompson, J.N. (1983). The embryonic cell lineage of the nematode *Caenorhabditis elegans*. Devl. Biol. 100, 64-119.
13. Tokumitsu, H., Takashi, N., Eto, K., Yano, S., Soderling, T.R., Muramatsu, M. (1999). Substrate Recognition by a Ca^{2+}/Calmodulin-dependent Protein Kinase Kinase. J. Biol. Chem. 274, 15803-15810.
14. Barstead, R.J., Waterson, R.H. (1988). The Basal Component of the Nematode Dense-body is Viniculin. J. Biol. Chem. 264, 10177-10185.
15. Fire, A., White-Harrison, S., Dixon, D. (1990). A modular set of lacZ fusion vectors for studying gene expression in *Caenorhabditis elegans*. Gene 93, 189-198.
16. Seydoux, G., Fire, A. (1995). Whole-Mount in Situ Hybridization for the Detection of RNA in *Caenorhabditis elegans* Embryos. Meth. Cell Biol. 48, 323-337.
17. Birchall, P.S., Fishpool, R.M., Albertson, D.G. (1995). Expression patterns of predicted genes from the *C. elegans* genome sequence visualized by FISH in whole organisms. Nature Genetics 11, 314-320.
18. Liao, V., Freedman, J.H. Personal communication.

19. Janse, G., Hazendonk, E., Thijssen, K.L., Plasterk, R.H.A. (1997) Reverse genetics by chemical mutagenesis in*Caenorhabditis elegans*. Nature Genetics 17, 119-121.
20. A description of this ongoing project is available on the internet at: http://www.sanger.ac.uk/Projects/C_elegans/Knockout/
21. Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E., Mello, C.C. (1998) Potent and specific interference by double stranded RNA in*Caenorhabditis elegans*. Nature 391, 806-810.
22. Shi, Y., Mello, C. (1998). A CBP/p300 homolog specifies multiple differentiation pathways in *Caenorhabditis elegans*. Genes & Dev. 12, 943-955.
23. Kostrouchova, M., Krause, M., Kostrouch, Z., Rall, J.E. (1998). CHR3: a*Caenorhabditis elegans* orphan nuclear hormone receptor required for proper epidermal development and molting. Development 125, 1617-1626.
24. Database searches performed using WU-BLAST 2.0 accessed at the following internet server: http://www.sanger.ac.uk/Projects/C_elegans/blast_server.shtml. Also see Gish, W., States, D.J. (1993). Identification of protein coding regions by database similarity search. Nature Genetics 3, 266-272.
25. Glenney, J.R. Jr., Weber, K. (1983). Detection of calmodulin-binding polypeptides separated in SDS-polyacrylamide gels by a sensitive [125I]calmodulin gel overlay assay. Methods Enzymol. 102, 204-210.
26. Haribabu, B., Hook, S.S., Selbert, M.A., Goldstein, E.G., Tomhave, E.D., Edelman, A.M., Snyderman, R., Means, A.R. (1995). Human calcium-calmodulin dependant protein kinase I: cDNA cloning, domain structure and activation by phosphorylation at threonine 177 by calcium-calmodulin dependent protein kinase I kinase. EMBO 14, 3679-3686.
27. Fukunaga, R., Hunter, T. (1997). MNK1, a new MAP kinase activated protein kinase, isolated by a novel expression screening method for identifying protein kinase substrates. EMBO J. 16, 1921-1933.
28. Kane, C.D., Hook, S.S., Means, A.R. Manuscript in preparation.